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ORGANOTIN-INDUCED HEMOLYSIS, SHAPE TRANSFORMATION AND INTRAMEMBRANOUS AGGREGATES IN HUMAN ERYTHROCYTES

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Organotin compounds examined in this study exhibited a relative order of potency for induction of in vitro *hemolysis in human erythrocytes as follows: tri-n-butyltin > tri-n-propyltin > tetra-n-butyltin > triphenyltin chloride > tri-n-ethyltin bromide > dibutyltin dichloride > stannous chloride > tri-n-methyltin chloride = butyltin chloride dihydroxide. All of the organotin compounds induced erythrocyte shape transformation from the normal discocyte to an echinocyte and, in addition, triphenyltin chloride, tetra-n-butyltin and tri-n-ethyltin bromide also elicited stomatocyte formation at higher concentrations. Select organotin compounds also formed tin-containing aggregates within the plasma membrane. The relative order of effectiveness for organotin induction of intramembranous aggregates was tri-n-butyltin > tri-n-propyltin > tetra-n-butyltin > tri-n-ethyltin bromide, which was based upon the lowest concentration at which they were observed. These results support the previously suggested theory that organotins are membrane effectors because of their comparatively high hydrophobic, lipid partitioning properties. The relatively lipophilic compound, triphenyltin chloride, appeared to be anomalous because it did not readily promote hemolysis or induce the formation of intramembranous aggregates in human erythrocytes. A log-linear statistical model demonstrated an association of hemolysis with both tri-n-butyltin aggregate formation and shape transformation. Select organotin compounds should be useful probes in membrane studies because of their numerous effects.*

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2. Key words: intramembranous aggregates, organotins, shape transformation, tributyltin, triethyltin, tripropyltin.

3. Abbreviations: DBT, dibutylin dichloride; MBT, butyltinchloride dihydroxide; $SnCl_2$, stannous chloride; TBT, tri-n-butyltin; TET, tri-n-ethyltin bromide; TMT, tri-n-methyltin chloride; TPhT, triphenyltin chloride; TPT, tri-n-propyltin; TTBT, tetra-n-butyltin.

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INTRODUCTION

Organotin compounds produce membrane effects such as inhibition of chemotaxis (Arakawa and Wada, 1984), uncoupling of oxidative phosphorylation (Aldridge and Cremer, 1955), shutdown of the sodium-potassium and calcium pumps (Selwyn, 1976), and membrane lysis (Yoshikawa and Ishii, 1962). Select organotin compounds induce membrane lysis at low concentrations (Byington et al., 1974). Tri-n-butyltin (TBT) is a very effective hemolytic agent, causing membrane leakage at 5 μM concentrations (Gray et al., 1986). Other trialkyltin compounds are also effective membrane toxicants and their relative potency correlates well with molecular lipophilicity (Wong et al., 1982; Wulf and Byington, 1975). Hydrophobicity of individual organotin compounds is a primary factor regulating relative toxicity. Organotin compounds examined in the following study were employed because they have differing lipid solubilities based on numbers of butyl chains or numbers of carbon atoms in alkyl chains.

TBT has been shown to produce 60-70 nm diameter, tin-containing aggregates in cell membranes at or above 10 μM concentrations (Porvaznik et al., 1986). Intramembranous TBT aggregates appear to be analogous to vesicles formed in aqueous solutions by numerous hydrophobic compounds (Fendler, 1983). Didodecyldimethylammonium compounds form vesicles spontaneously in water, and vesicle sizes can be altered by changing the anion present (Talmon et al., 1983). The forces stabilizing these vesicles are under active investigation and it appears that a hierarchy of attractive forces operates between hydrophobic residues (Pashley et al., 1985). Intramembranous TBT aggregates also vary in size, depending upon the anions present, in a fashion similar to size changes observed in aqueous solutions of didodecyldimethylammonium vesicles. Intramembranous TBT aggregates observed in cyanide-treated or 2,3-dimercapto-1-propanol-treated erythrocytes are significantly larger than those observed in control-treated preparations (Gray et al., 1986). TBT is the first known xenobiotic compound to form stable aggregates in membranes (Porvaznik et al., 1986). One purpose of the present study was to examine whether additional organotin compounds form similar, intramembranous aggregates and, if they are formed, the concentrations necessary for that formation.

TBT also induces shape transformation in human erythrocytes at 0.1 μM, forming echinocytes from discocytes (Porvaznik et al., 1986). The coupled bilayer membrane model predicts that echinocytic compounds such as TBT preferentially partition into the outer leaflet of the lipid bilayer (Sheetz and Singer, 1974). Echinocytic transformation is usually induced by amphipathic anions or nonionized compounds (Deuticke, 1968). Compounds that induce cup-shaped stomatocytes are thought to partition into the inner leaflet of the lipid bilayer because they are cationic (Deuticke, 1968). Equilibrium sequestration of cationic, anionic, or neutral hydrophobic compounds in either the inner or outer leaflets of the lipid membrane bilayer is attributed to the organization of different lipid constituents in each half of the bilayer (Sheetz and

Singer, 1974). Complicating examples of compounds that induce echinocyte transformation followed by discocyte recovery may be explained by a nonequilibrium state (Sheetz and Singer, 1976). A recently proposed model for human erythrocyte shape transformation suggests that cell shape depends not only on properties of lipids in the bilayers but also on interactions of membrane and cytoskeletal proteins with those lipid constituents (Kuypers et al., 1984). Polyphosphoinositol is one example of a lipid cofactor required for linkage of cytoskeletal proteins to the transmembrane protein, glycophorin (Anderson and Marchesi, 1985). Clearly, drug-induced erythrocyte shape transformation involves complex membrane interactions at the molecular level (Morrow and Anderson, 1986). A second purpose of the following investigation is to observe erythrocyte shape transformations in conjunction with hemolysis profiles induced by select organotin compounds at various concentrations. Lastly, a causal model is statistically developed using measurable or observable factors such as organotin aggregates within membranes, dose or concentration of organotin compound, erythocyte shape transformation, and hemolysis to show likely associations that exist between those factors.

METHODS

Chemicals and Reagents. TBT (lot 9118ek) and triphenyltin chloride (TPhT) (lot 04421EM) were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Tri-n-propyltin (TPT) (lot 71122), tetra-n-butyltin (TTBT) (lot 111683), tetra-n-ethyltin (TET) (lot 71135), dibutyltin dichloride (DBT) (lot 71127), tri-n-methyltin chloride (TMT) (lot 71166) and butyltinchloride dihydroxide (MBT) (lot 18594) were purchased from Alfa Products, Thiokol/Ventron Division, Danvers, MA.

Stannous chloride ($SnCl_2$) was obtained from the J.T. Baker Chemical Co., Phillipsburg, NJ. MBT and stannous chloride were added as solids to erythrocyte suspensions. Stock solutions of each of the other compounds were prepared in ethanol. Appropriate dilutions were added to erythrocyte suspensions to produce desired concentrations.

Erythrocyte Hemolysis and Microscopy. Erythrocyte collection and hemolysis assays were performed in accordance with previously described methods (Gray et al., 1986). Sample preparation and electron microscopy were performed as previously described (Porvaznik et al., 1986). An Olympus IMT-2 inverted research microscope, manufactured by Olympus Optical Co. Ltd., Tokyo, Japan, was used for light microscopy of erythrocyte suspensions. A 0.1 ml volume was removed from each erythrocyte suspension and added to 1.0 ml of Dulbecco's suspending medium in a 35-mm-style plastic culture dish (Flow Laboratories, McLean, VA). Dishes were placed on the inverted microscope stage and examined by phase contrast optics. The erythrocyte suspensions were examined at $\times$ 300 magnification, and the center field of each plate was photographed using an OM-2S camera.

Statistical Analysis. Percent hemolysis for each compound was analyzed by a repeated measures design using BMDP4V software (Dixon, 1983). The between factor was dose, and the within factor was hours (Barcikowski, 1983). Multiple comparisons of data were performed using a Scheffe technique (Barcikowski, 1983).

A log-linear model was used to examine the relationships between hemolysis, shape transformation, intramembranous tin-containing aggregates, compound, and dose (Bishop et al., 1975). The analysis began (model one) by assuming no associations. All pairwise associations were determined and their corresponding statistics (degrees of freedom and chi-square) were subtracted from model one. The pairwise association that had the smallest of all significant probabilities ($P < 0.05$) became model two. Using model two as the new base model, other pairwise associations were formed and the associated statistics (degrees of freedom and chi-square) were subtracted from model two. The model with the smallest significant probability was chosen as model three. Model building continued until there were no more significant probabilities. The above procedures were conducted using BMDP4F software (Dixon, 1983).

The hemolysis category at each dose was established by using the mean at 4 hr. The mean differed significantly ($p < 0.05$) from the zero dose at 4 hr for all but the first category. The four established categories for hemolysis were 1 - slight ($< 4\%$ and $P < 0.05$), 2 - mild ($< 25\%$ and $P < 0.05$), 3 - moderate ($> 25\%$ and $> 75\%$, and $p < 0.05$), and 4 - severe ($> 75\%$ and $p < 0.05$).

RESULTS

Figures 1-6 show hemolysis kinetic profiles for human erythrocytes induced by the six organotins examined in these studies. The relative order of potency for induction of *in vitro* hemolysis in human erythrocytes is TBT > TPT > TTBT ≥ TPht > TET > DBT > $SnCl_2$ > TMT = MBT. Both TET (Fig. 1) and TPT (Fig. 2) seem to induce sigmoidal patterns similar to that observed with TBT (Fig. 3). These results contrast those obtained with TTBT (Fig. 4), TPhT (Fig. 5), and DBT (Fig. 6). Figure 4 indicates a more hyperbolic pattern with 5000 μM TTBT significantly reducing hemolysis at 0.5 hr compared to a 1000 μM dose ($p < 0.0001$). The TPhT hemolysis kinetic profiles at 100 and 1000 μM show prominent early lag phases characteristic of sigmoidal patterns. The DBT profile was more hyperbolic (Fig. 6).

Hemolysis data were statistically analyzed and values categorized as defined above in the Methods section. TBT at 25 μM produced mild hemolysis at 0.5 hr ($p < 0.0001$), moderate hemolysis at 1 hr ($p < 0.0001$), and severe hemolysis after 1.5 hr ($p < 0.0001$). At a 10-μM dose, TBT induced mild hemolysis at 1 hr ($p = 0.0025$), 1.5 hr ($p < 0.0001$), and 2 hr ($p < 0.0001$). Moderate hemolysis was observed at 3 and 4 hr ($p < 0.0001$) after 10-μM TBT treatment. Treatment with 5-μM TBT produced mild hemolysis at 3 hr ($p = 0.0353$) and 4 hr ($p = 0.0059$). At 150 μM, TPT induced mild hemolysis at 0.5 hr ($p = 0.0006$), moderate hemolysis at 1 hr ($p < 0.0001$), and severe

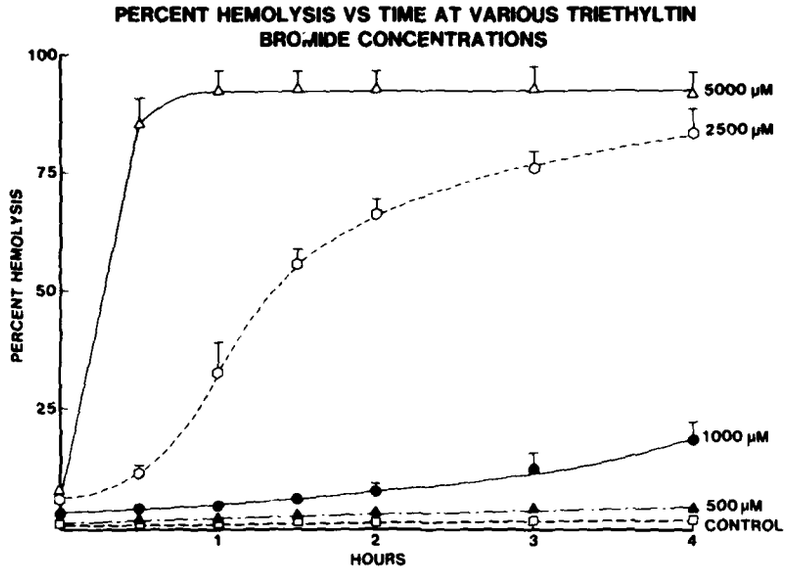

FIGURE 1. Percent hemolysis as a function of time for various TET concentrations. Cell suspensions had about 2.3×10^8 erythrocytes per milliliter plus 1% ethanol. Each data point is the mean of five determinations with an attached standard deviation bar. (□----□) 171 mM ethanol control, (▲- - -▲) 500 μM, (●——●) 1000 μM, (◇----◇) 2500 μM, (Δ——Δ) 5000 μM.

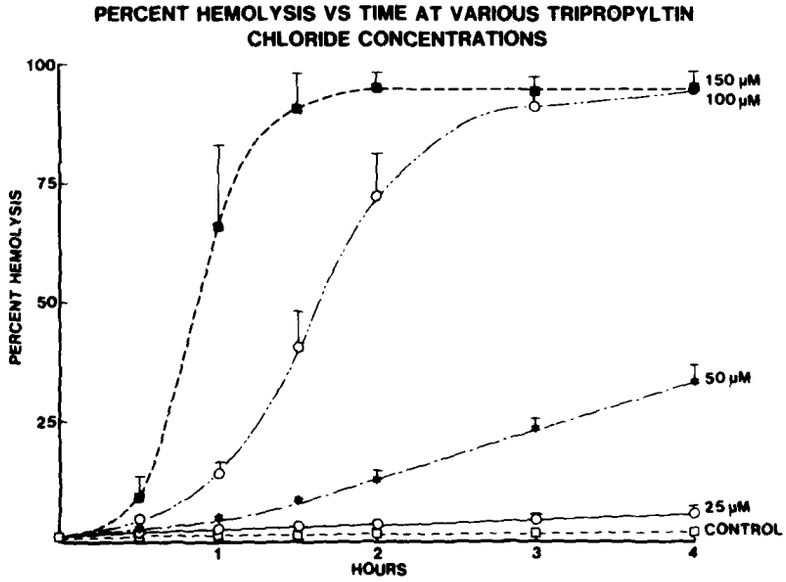

FIGURE 2. Percent hemolysis as a function of time for various TPT concentrations. Cell suspensions had about 2.3×10^8 erythrocytes per milliliter plus 1% ethanol. Each data point is the mean of five determinations with an attached standard deviation bar. (□----□) 171 mM ethanol control, (○——○) 25 μM, (*——*) 50 μM, (○- - -○) 100 μM, (■----■) 150 μM.

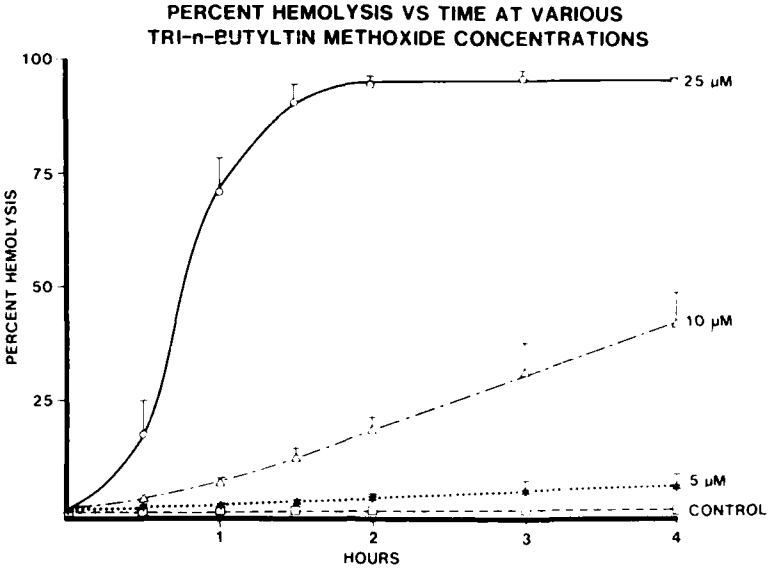

FIGURE 3. Percent hemolysis as a function of time for various TBT concentrations. Cell suspensions had about 2.3×10^8 erythrocytes per milliliter plus 1% ethanol. Data points with attached standard deviation bars are shown. Controls, 10 μM, and 25 μM had n values of 52, and 5 μM had an n of 5 (□----□) 171 mM control, (*•••••*) 5 μM, (Δ—-—Δ) 10 μM, and (○——○) 25 μM.

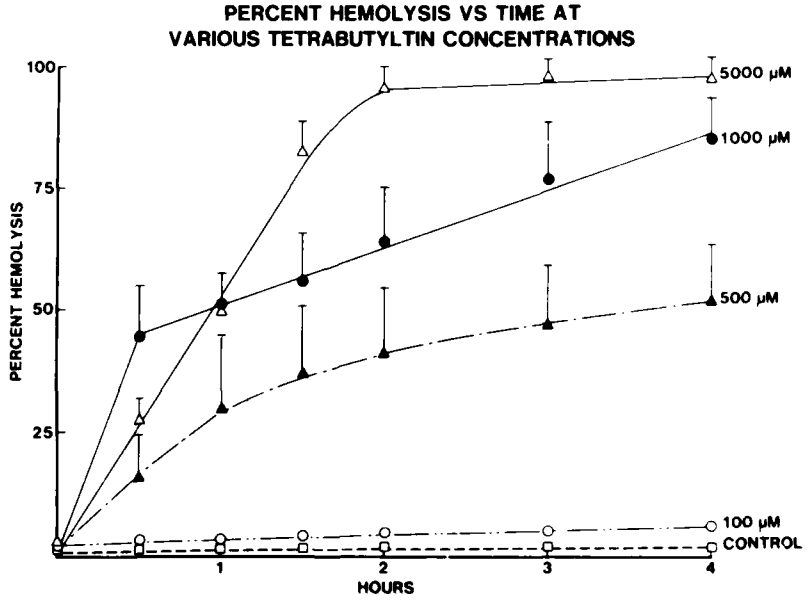

FIGURE 4. Percent hemolysis as a function of time for various TTBT concentrations. Cell suspensions had about 2.3×10^8 erythrocytes per milliliter plus 1% ethanol. Each data point is the mean of five determinations with an attached standard deviation bar. (□----□) 171 mM ethanol control, (○—··—○) 100 μM, (▲—-—▲) 500 μM, (●——●) 1000 μM, (Δ——Δ) 5000 μM.

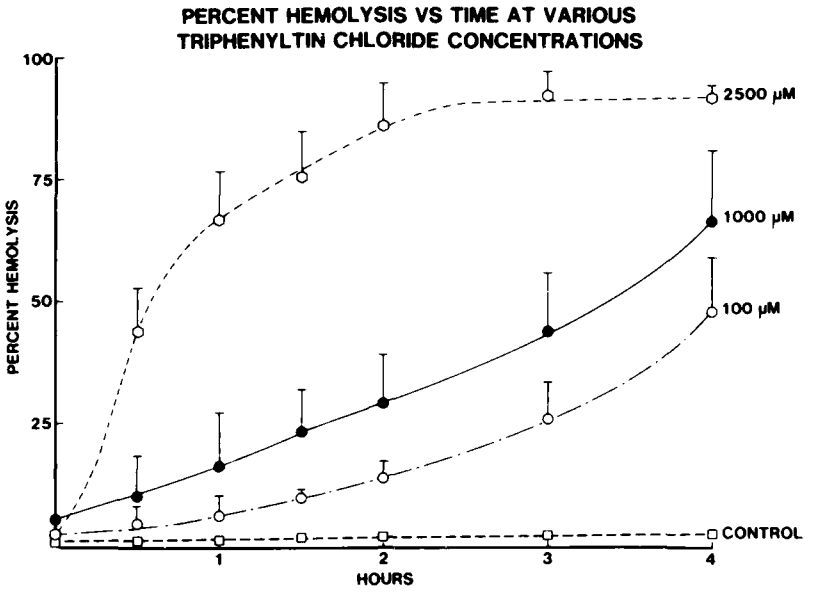

FIGURE 5. Percent hemolysis as a function of time for various TPhT concentrations. Cell suspensions had about 2.3×10^8 erythrocytes per milliliter plus 1% ethanol. Each data point is the mean of five determinations with an attached standard deviation bar. (□----□) 171 mM ethanol control, (○——○) 100 μM, (●——●) 100 μM, (○---○) 2500 μM.

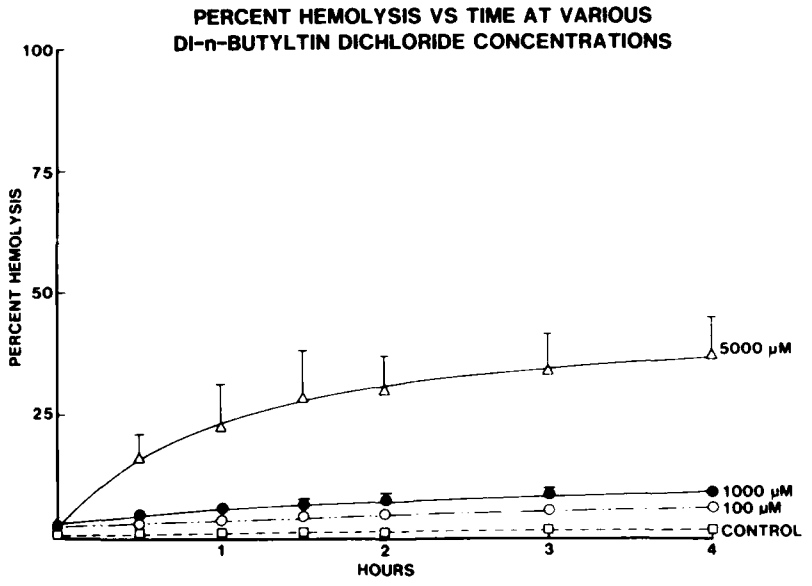

FIGURE 6. Percent hemolysis as a function of time for various DBT concentrations. Cell suspensions had about 2.3×10^8 erythrocytes per milliliter plus 1% ethanol. Each data point is the mean of five determinations with an attached standard deviation bar. (□----□) 171 mM ethanol control, (○----○) 100 μM, (●——●) 1000 μM, (△——△) 5000 μM.

hemolysis at 1.5 hr or longer ($p < 0.0001$). A dose of 100 μM TPT caused mild hemolysis at 1 hr ($p < 0.0001$), moderate hemolysis at 1.5 and 2 hr ($p < 0.0001$), and severe hemolysis at 3 and 4 hr ($p < 0.0001$). At a concentration of 50 μM, TPT produced mild hemolysis at 1.5 hr ($p = 0.0101$), 2 hr, and 3 hr ($p < 0.0001$), plus moderate hemolysis at 4 hr ($p < 0.0001$). A dose of 5000 μM TTBT induced moderate hemolysis at 0.5 and 1 hr ($p < 0.0001$) and severe hemolysis from 1.5 to 4 hr ($p < 0.0001$). TTBT at 1000 μM caused moderate hemolysis from 0.5 to 2 hr and severe hemolysis at 3 and 4 hr ($p < 0.0001$), and 500 μM TTBT resulted in mild hemolysis at 0.5 hr ($p < 0.0001$) and moderate hemolysis from 1 to 4 hr ($p < 0.0001$). TPhT at 2500 μM elicited moderate hemolysis at 0.5 and 1 hr ($p < 0.0001$) and severe hemolysis from 2 to 4 hr ($p < 0.0001$), and 1000 μM TPhT produced mild hemolysis from 0.5 to 1.5 hr and moderate hemolysis from 2 to 4 hr ($p < 0.0001$). A dose of 500 μM TPhT elicited mild hemolysis from 0.5 to 2 hr ($p < 0.0001$) and moderate hemolysis at 3 and 4 hr ($p < 0.0001$), and TPhT at 100 μM produced mild hemolysis at 2 hr ($p = 0.0371$) and moderate hemolysis at 3 and 4 hr ($p < 0.0001$). TET at 5000 μM elicited severe hemolysis from 0.5 to 4 hr ($p < 0.0001$), and TET at 2500 μM produced mild hemolysis at 0.5 hr ($p < 0.0001$), moderate hemolysis at 1 and 2 hr ($p < 0.0001$), and severe hemolysis at 3 and 4 hr ($p < 0.0001$). A 1000-μM TET dose caused mild hemolysis from 1 to 4 hr ($p < 0.01$). DBT at 5000 μM induced moderate hemolysis from 1.5 to 4 hr ($p < 0.0001$). Mild hemolysis was produced by DBT at 100 μM (4 hr, $p = 0.0111$), 500 μM (1.5 to 4 hr, $p < 0.01$), 1000 μM (1 to 4 hr, $p < 0.01$) and 5000 μM (0.5 and 1 hr, $p < 0.0001$). TMT was not hemolytic at any tested concentration.

Erythrocyte shape transformation was induced by every organotin compound examined, but the responses varied remarkably between low and high concentrations, as shown in Table 1. Figure 7a shows a photomicrograph of a control erythrocyte

TABLE 1
Erythrocyte Shape Transformation by Organotin Compounds

	Concentration and Shape Transformation											
Organotin	0	0.1	1.0	10	25	50	100	150	500	1000	2500	5000
TMT	MC	NE	NE	NE	NE	NE	MC	NE	E	E	E	E
TET	MC	NE	NE	NE	NE	NE	E	NE	E	E	S	S
TPT	MC	NE	NE	E	E	E	E	E	NE	NE	NE	NE
TBT	MC	RE	E	E	E	NE	E	NE	NE	NE	NE	NE
TTBT	MC	NE	NE	NE	RE	NE	E	NE	E,C	S	NE	S
DBT	MC	NE	NE	NE	E	NE	C	NE	C	C	NE	C
TPhT	MC	NE	NE	E	NE	NE	S	NE	S	S	S	NE

MC Marginal crenation of discocyte due to 1% ethanol vehicle.
NE Not examined at this concentration.
RE Cells reversibly transform to spherical echinocytes but fail to hemolyze.
C Clumping.
E Echinocyte.
S Stomatocyte.

preparation treated with 171 mM ethanol (organotin vehicle). Most of the cells are discocytes with occasional marginal crenation produced by the 1% ethanol vehicle. Figure 7b demonstrates the extensive erythrocyte clumping induced by 1000 μM DBT. Cell aggregation is the most pronounced effect of DBT treatment (Table 1) and occurs infrequently with TTBT. Echinocyte formation was the most frequent response elicited by nearly all of the organotins examined. Figure 7c shows the effect of 1000 μM TMT in producing echinocyte shape transformation. Stomatocyte formation was the most consistent finding in erythrocytes exposed to TPhT (Fig. 7d) and to higher concentrations of TTBT ($\geq$ 1000 μM) or TET ($\geq$ 2500 μM). TTBT induced echinocytes at 100 μM, clumping at 500 μM, and stomatocytes at higher concentrations (Fig. 7e).

Table 2 lists concentrations at which organotin compounds produce membrane-intercalated aggregates observed by transmission electron microscopy. At concentrations up to 5000 μM, neither MBT nor tin chloride produced aggregates. The relative order of effectiveness for organotin induction of aggregate formation is TBT > TPT > TTBT > TET, based upon the lowest concentration at which they were observed. Clearly, hemolysis is induced by each of the organotins below the concentration required for aggregate formation (Figs. 1-4). Also, DBT and TPhT support hemolysis but fail to produce any recognizable membrane aggregate structures at 5000 and 2500 μM, respectively (Figs. 5 and 6).

Figures 7f-j are electron micrographs showing membrane-associated, tin-containing aggregates produced by different concentrations of various organotins. Control preparations with 171 mM ethanol lack membrane aggregates (Fig. 7f). Intramembranous aggregates are visible in erythrocyte membranes at a dose of 5000 μM TET (Fig. 7g). Figures 7h-j demonstrate aggregates associated with erythrocyte membranes treated with 50 μM TPT, 100 μM TBT, and 1000 μM TTBT, respectively. Membrane aggregates are also observed in erythrocyte preparations of TPT, TBT, and TTBT at concentrations greater than the minimum listed (Table 2).

Table 3 lists results for model studies using variables listed in these experiments. The only variables found to be statistically associated in all organotin compounds at all doses were intramembranous tin aggregates with hemolysis ($p = 0.0048$) and shape transformation with hemolysis ($p = 0.0268$).

DISCUSSION

The molecular mechanisms for organotin-induced membrane disruption are not yet fully understood. The kinetic profiles for TET-, TPT-, and TBT-induced hemolysis show a complex series of steps culminating in loss of membrane barrier function. Each of the above three trialkyltins induce echinocyte formation at modest concentrations, indicating relatively rapid incorporation into the outer membrane leaflet of the erythrocyte (Deuticke, 1968; Sheetz and Singer, 1974). Indeed, at sufficiently high

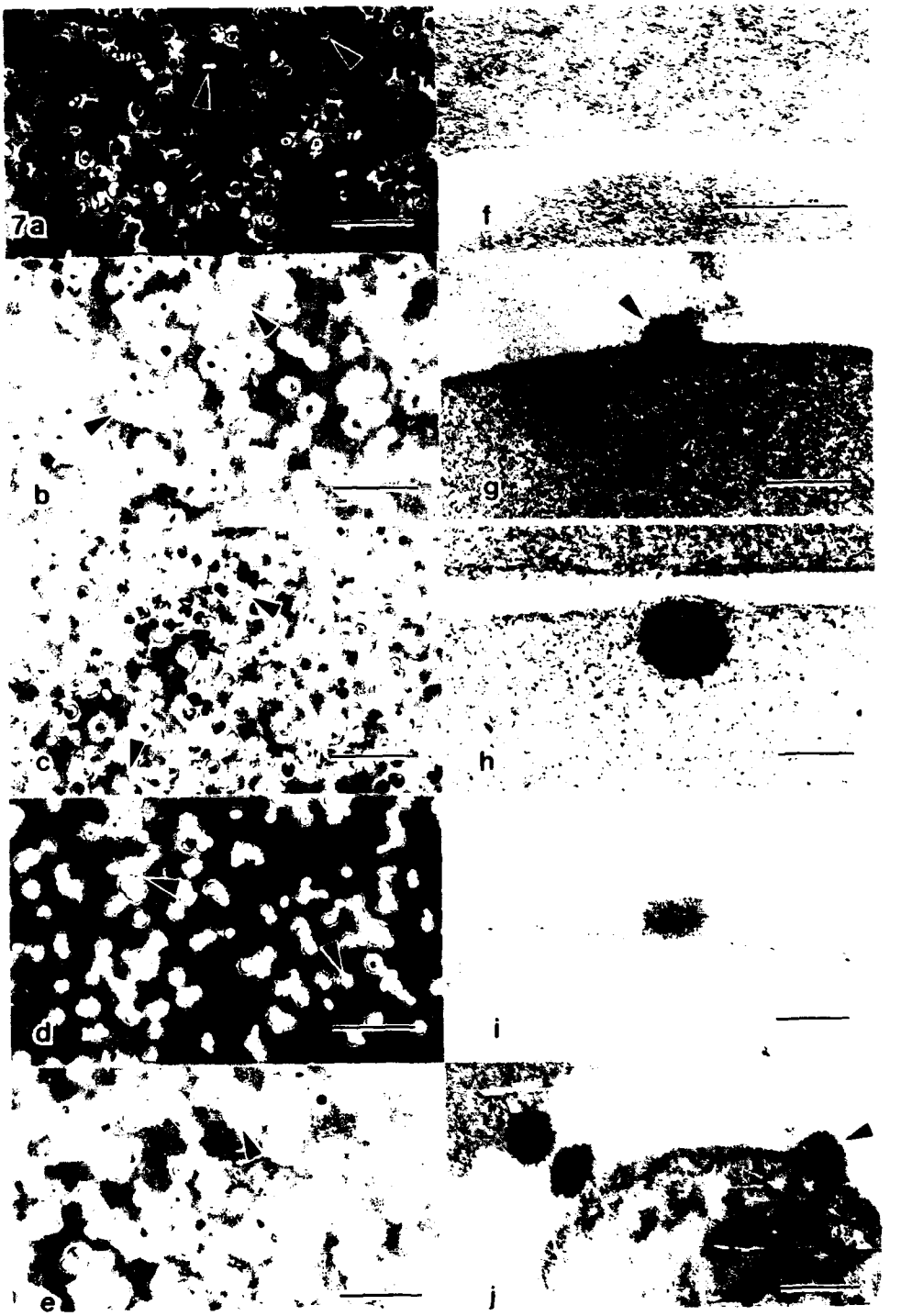
7a
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TABLE 2
Intramembranous Aggregate Formation Induced by Organotin Compounds

	Concentration (μM)											
Organotin	0	0.1	1.0	10	25	50	100	150	500	1000	2500	5000
TMT	–	NE	NE	NE	NE	NE	–	NE	–	–	–	–
TET	–	NE	NE	NE	NE	NE	–	NE	–	–	–	+
TPT	–	NE	NE	–	–	+	+	+	NE	NE	NE	NE
TBT	–	–	–	+	+	+	+	NE	NE	NE	NE	NE
TTBT	–	NE	NE	NE	–	NE	–	NE	–	+	NE	+
DBT	–	NE	NE	NE	–	NE	–	NE	–	–	NE	–
TPhT	–	NE	NE	–	NE	NE	–	NE	–	–	–	NE

– Aggregates not observed.
+ Aggregates observed.
NE Not examined at this concentration.

concentrations, aggregates of each of these trialkyltin compounds are visible in erythrocyte membranes (Fig. 7). However, TMT did not form membrane-associated structures at any concentration examined. TMT did induce echinocyte formation, but, even at 5000 μM, TMT treatment did not result in significant hemolysis at 4 hr. These observations support the contention that trialkyltins are membrane effectors by virtue of their hydrophobic, lipid partitioning properties (Davis and Smith, 1982;

FIGURES 7 a-e. Phase contrast micrographs of human erythrocyte suspensions incubated at 37° with organotin compounds. The scale bar represents 50 μm in each photomicrograph. (a) Most of the cells in this 30-min 1% ethanol-treated control preparation are typical biconcave discocytes (arrows) with very few crenated forms. (b) Cell clumping (arrows) is the most pronounced effect 30 min after 1000-μM DBT treatment of red blood cells (RBCs). (c) Incubation for 3 hr with 1000 μM TMT induces crenated discocytes or spherical echinocytes (arrows). (d) A 5-min incubation with 1000 μM TPhT produces numerous cup-shaped stomatocytes plus aggregations (arrows). (e) A 30-min incubation with 1000 μM TTBT induces stomatocytes and spherostomatocytes (arrow).

FIGURES 7 f-j. Electron micrographs of human erythrocyte suspensions incubated at 37° with organotin compounds. The scale bar represents 200 nm in f and 100 nm in g-j. (f) A 1% ethanol-treated control preparation lacks any electron-dense membrane-associated aggregates or any extruded hemoglobin in the extracellular space. (g) A 5000-μM TET preparation sampled 30 min after addition of organotin. The arrow points to a membrane-associated electron density characteristic of this TET concentration. (h) A preparation sampled 30 min after addition of 50 μM TPT. The membrane-associated electron densities are rarely observed protruding into cells as shown in this micrograph. (i) Red blood cells were treated with 100 μM TBT for 15 min, producing electron-dense aggregates. (j) Red blood cells treated with 5000 μM TTBT for 30 min. Membrane-associated aggregates (arrow) persist even after lysis and hemoglobin extrusion.

Porvaznik et al., 1986; Selwyn, 1976). The detailed molecular steps required for lysis following incorporation of alkyltin compounds into membranes remain unclear. Membrane-associated alkyltin aggregates are not required for membrane disruption, although their presence is indicative of a concentration of alkyltin that will result in membrane lysis.

TTBT displays a more hyperbolic hemolytic kinetic pattern than the other trialkyltin compounds examined. The finding that the percent hemolysis at 0.5 hr for 5000 μM TTBT was less than the value at 1000 μM indicates membrane interactions different from those of any of the other organotins examined. Both TTBT concentrations induce stomatocyte formation rather than echinocyte formation at 500 μM. Stomatocytes are considered more stable cellular forms than echinocytes (Palek et al., 1974). The observation that stomatocytes appear above 1000 μM and predominate at 5000 μM may indicate nonequilibrium partitioning between outer and inner leaflets of the membrane bilayers (Sheetz and Singer, 1976). In addition, intramembranous tin aggregates were not observed at 500 μM TTBT, but were observed at 1000 μM. Aggregate formation may indicate saturating conditions in both the aqueous and lipid compartments. Organotin saturation may permit sufficient localization in inner bilayers to promote stomatocyte formation and account for the decreased percent hemolysis at 0.5 hr for 5000 μM TTBT treatment.

Human erythrocytes transform into stomatocytes at a dose of 100 μM TPhT, and this compound is the most interesting organotin examined because it is much less hemolytic in human erythrocytes compared to results with other species (Byington et al.,

TABLE 3
Five-Factor Association Model in Organotin-Exposed Erythocytes

Model Number	Model Proposed	Degrees of Freedom	Chi-square	Probability Statistic
1	d, c, a, s, h (no association)	3665	583.67	1.0000
2	d, c, ah, s (step one)	3605	570.75	1.0000
1-2	difference	60	12.92	0.0048
3	d, c, ah, vh (step two)	3647	543.51	1.0000
2-3	difference	15	27.24	0.0268

d = dose.
c = compound.
a = aggregates.
s = shape.
h = hemolysis.
sh = association between shape and hemolysis.
ah = association between aggregates and hemolysis.

1974). The human erythrocyte membrane must differ sufficiently from hog, dog, rat, or rabbit red cell membranes to account for this difference. The absence of membrane-associated TPhT aggregates in human erythrocytes is unexpected since the compound has a relatively high octanol-water partition coefficient (Wong et al., 1982; Wulf and Byington, 1975). The human erythrocyte inner leaflet may accommodate sufficient TPhT to reduce concentrations below the aggregation level. Alternatively, TPhT is a stronger Lewis acid than either TMT, TET, TPT, or TTBT (Davis and Smith, 1982). Perhaps this electron affinity of TPhT prevents aggregate formation or interferes with its fixation or staining with osmium tetroxide, which is necessary for electron microscopic observation.

These studies demonstrate that human erythrocytes exposed to sufficiently high concentrations of certain organotins present electron-dense aggregates within plasma membranes. Relatively water-soluble compounds, such as MBT, DBT, and TMT, do not form intramembranous structures in aqueous suspending media. These and other more water-soluble compounds probably do not aggregate into structures that sequester in membranes (Fendler, 1983). Lipophilic compounds that do form intramembranous aggregates will also produce structures stabilized by osmium tetroxide fixation that may be observed by transmission electron microscopy. Osmication replaces tin in these samples, and ethanol dehydration removes tin from unfixed samples by dissolving lipophilic organotins from materials prepared by routine fixation and dehydration (Porvaznik et al., 1986). Electron-dense, tin-containing intramembranous aggregates may be preserved using glutaraldehyde-carbohydrazide embedding, without the usual procedures employed with plastic resins (Porvaznik et al., 1986).

The detailed molecular mechanisms for organotin-induced erythrocyte membrane effects from shape transformation through hemolysis remain enigmatic. One factor known to govern organotin-mediated membrane effects is the relative lipophilicity of the individual compounds (Gray et al., 1986; Laughlin et al., 1985; Wong et al., 1982; Wulf and Byington, 1975). It is obvious that compounds capable of entering membrane lipid domains fulfill a prerequisite for being membrane effectors. Organotin-induced inhibition of sodium-potassium and calcium pumps may destabilize membranes and deplete cellular ATP (Morrow and Anderson, 1986; Selwyn, 1976). ATP depletion leads to echinocyte transformation which is reversible for several hours upon ATP restoration in erythrocytes (Nakao et al., 1960). Anion transport across erythrocyte membranes mediated by organotins could also contribute to membrane instability (Anderson and Marchesi, 1985; Arakawa and Wada, 1984; Deuticke, 1968; Morrow and Anderson, 1986; Wieth and Tosteson, 1979; Wulf and Byington, 1975). Indeed, the suggestion that selected organotins could strip anionic polyphosphoinositol phospholipids from the transmembrane protein glycophorin, resulting in destabilization of the bond to cytoskeletal protein 4.1, merits experimental consideration (Morrow and Anderson, 1986).

The log-linear analysis indicates four causal models relating shape transformation (S), hemolysis (H), and tin-containing aggregates within membranes (A).

1. S → H → A
2. A → H → S
3. H → S, H → A
4. S → H, A → H

The fourth model fits the data and observations presented here. Shape transformation and organotin aggregation occur independently of each other and before hemolysis can proceed. Light and electron microscopic observations of treated erythrocytes support the idea of independent processes of shape transformation and organotin aggregation leading to hemolysis. It is interesting that data analysis demonstrated no association between tin-containing aggregates and shape transformation, or between tin-containing aggregates and concentration.

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